METHOD AND TEST STRIPS FOR THE MEASUREMENT OF FAT LOSS DURING WEIGHT LOSS PROGRAMS

The present invention affords a method and test strips for monitoring the effectiveness of a diet, or a diet plus exercise regimen (whether undertaken for a therapeutic purpose or for improving appearance), which specifically provides information on body fat loss and is usable by members of the general public on samples such as urine or saliva.

Patent Application

This application is a continuation-in-part of U.S. application Serial No. 10/067,660 filed February 4, 2002.

Background of the Invention

Obesity of human beings is an ever-increasing source of medical concern, especially in the United States, today. It is estimated that over 60% of the U.S. population is obese. Studies have shown obesity to be the leading cause of diabetes, hypercholesterolemia and other disorders that lead to kidney and liver failure. A recently issued, widely commented upon, study attributes failure to diagnose many cancers at an early time, when effective treatment could be given, to serious obesity. Obesity likewise accounts for, or at least plays a part in, many severe heart failures.

Numerous diets employing special nutrient preparations or food choice prescriptions are available and widely used, e.g. Weight-watcher's, NutriSystem, New Beverly Hills, Jenny Craig, the Atkins diet, the Pritikin Principle diet, etc. Many of these diets are helpful in

effecting weight loss, but the sad fact is that the majority of people who experience such weight loss gain it back, in many instances gaining even more than they lost, within a relatively short time period.

People who are on a diet (or diet plus exercise) regimen, are prone to monitor their own progress by total weight loss, as measured on weighing scales. It has long been known, however, that for meaningful weight loss, body fat must be diminished on a steady basis and this cannot be measured by simple weighing because body fluid balance in particular, and perhaps other factors, play an ever-fluctuating and presently unmeasurable role in total body weight.

In order to lose bodily fat, it has long been known that the fat must be metabolized into smaller molecules known as ketone bodies. Total ketone bodies in humans have three components: acetone, acetoacetate and β-hydroxybutyrate. Of these components, acetone and acetoacetate together normally comprise at most 20 - 25% or less of total ketone bodies, with acetone alone usually being present in less than 2% of total ketone bodies and β-hydroxybutyrate, comprising at least 75% and often 80% or slightly more of total ketone bodies. Methods for measuring acetone and acetoacetate, particularly for people afflicted with Type 1 diabetes, where substantial amounts of this mixture are produced, have long been known. In this regard, acetone, which is a low molecular weight, highly volatile and hence unstable, material, and is present in total ketone bodies of any individual in very low concentration, is very seldom measured alone. Instead, acetoacetate and acetone are usually measured together using a nitroprusside reaction under alkaline conditions, in blood or urine samples. The reaction produces an intense purple color, wherein the intensity of the color is

regarded as varying in rough proportion to the concentration of acetoacetate in the sample. U.S. Patents 3,212,855 to Mast et al; 4,147,574 to Magers et al; and 4,440,724 to Tabb et al each illustrate methods and devices for measuring acetoacetate (which they refer to somewhat erroneously as "ketone bodies") in urine or serum, using this test scheme. In addition, so-called "ketone" test strips have been commercially available for many years from each of Bayer and Roche Diagnostics, and they measure acetoacetate solely.

While these strips do not, and cannot, measure β-hydroxybutyrate, the *primary* component of total ketone bodies, they have been used by Type I diabetics and they have been used successfully in some Atkins diet programs that comprise a high proportion of fatty foods and a very low amount of carbohydrates. Another patent involving test strips that measure acetone and acetoacetate is U.S. 5,260,219 to Fritz. An overall drawback of this type of strip is that, when utilized to test urine of people on conventional "balanced" weight loss diets of 1000-1500 calories per day, it is generally insensitive, producing no reaction or a very faint one.

It is important to note that, while acetone in a sample can be measured by including a nitroprusside or a diazonium salt in the mixture of chemicals assembled to accomplish the measurement, acetone is so high in volatility that a sample not subjected to measurement within 5 minutes or less of collection most probably will not have retained its original acetone content. This is normally true even if the sample is placed in a stoppered flask, since acetone easily volatilizes in the flask, and escapes rapidly when the stopper is removed.

Two patents describe two-step methods for measuring acetoacetate and β-hydroxybutyrate (but not acetone) in a sample, preferably of blood serum or plasma. In each

of them, the first step is to convert the acetoacetate in the sample to β-hydroxybutyrate. The second is to measure the total β-hydroxybutyrate content of the sample by converting it to another substance measurable at a known wave length. Both have methods have drawbacks which render them unsuitable for use by persons lacking laboratory training and/or availability of specialized instruments, in day-to-day monitoring of fat in weight loss regimens. The first of these, U.S. Patent 5,618,686 to Kojima *et al* must be conducted in an automated analyzer and requires different pH conditions in each of its two steps. In its first step, conducted at pH 7 to 7.5, acetoacetate is converted with the aid of reduced nicotinamide adenine dinucleotide (NADH) and β-hydroxybutyrate dehydrogenase to β-hydroxybutyrate, and in the second step, the quantity of β-hydroxybutyrate in the sample is measured. To make the measurement, both the hydroxybutyrate endogenous to the sample and the hydroxybutyrate obtained in the first step are converted into acetoacetate with β-hydroxybutyrate dehydrogenase under alkaline conditions at a pH above 8.5 and in the presence of excess nicotinamide adenine dinucleotide, with formation of reduced nicotinamide adenine dinucleotide in an amount that corresponds to the total hydroxybutyrate content as determined by measurement at a primary wavelength of 340 nm. This measurement is made in the automatic analyzer. While the method is accurate, it is clearly not adaptable to use by persons lacking laboratory training. It likewise cannot be converted to a dry chemistry format because the steps are incapable of being simultaneously performed because of their different pH requirements.

In the second of these two-step methods described in U.S. Patent 5,633,143 to Ueda et al, the first step of converting acetoacetate to β-hydroxybutyrate is accomplished in the same manner as in the first method, but the second step of measuring total β-hydroxybutyrate

requires the presence of a thiol derivative of nicotinamide adenine dinucleotide. The thiol derivative is incompatible with the dihydride of nicotinamide adenine dinucleotide that is used in the first step and care must be taken not to mix them. The reaction produces a yellow color at a wavelength of 400 nm. The color is hard to recognize with the naked eye and is easily interfered with if hemolysis occurs or bilirubin is present. To sum up, the method, albeit accurate when conducted by a well-trained technician, is not amenable to being used in a dry chemistry solid phase test device operable by persons without laboratory training.

Other methods of measuring β-hydroxybutyrate in biological fluids using the enzyme β-hydroxybutyrate dehydrogenase (HBD) in the presence of nicotinamide adenine dinucleotide (NAD) have long been known. In general, these methods produce reduced nicotinamide adenine dinucleotide (NADH) which can be measured at 340 nm. See Williamson et al, 1962, "Enzymatic Determination of D(-)- Hydroxybutyric Acid and Acetoacetate acid in Blood", *Biochem J. 82*: 90-96, Harano *et al*, 1990, "*Diabetic Care, 7*: 481-485; Harano et al,1990, "Development of Stable Film Test for Rapid Estimation of Blood or Plasma 3-Hydroxybutyrate, *Diabetic Care 13*: 522-524. There is also a Keto Site® test available from GDS Technology,Inc. of Elkhart, IN with a product insert dated 12/19/93. In this regard, see Tietz, *Textbook of Clinical Chemistry*, (Editors, Burtis and Ashwood, 1999) at pp 786-787. In all of these methods β-hydroxybutyrate dehydrogenase and NAD are caused to react with β-hydroxybutyrate in a blood sample. The reaction produces NADH which is measured with a tetrazolium salt in the presence of excess diaphorase enzyme at a pH in excess of 8.5. It produces a color that is proportional in intensity to the concentration of β-hydroxybutyrate in a blood sample. The reactions that occur are in two steps:

(a) β-hydroxybutyrate + NAD → Acetoacetate + NADH

(b) NADH + Tetrazolium dye → Reduced tetrazolium dye (purple color)

The KetoSite test cards which are available have been used mainly by diabetic patients who produce β-hydroxybutyrate as a result of severe insulin deficiencies which precipitate severe ketoacidosis. The cards are adapted only for use with blood samples but they were found to be capable of measuring β-hydroxybutyrate content of blood from persons on a low calorie diet.

Still other colorimetic methods for measuring β- hydroxybutyrate are known which employ Ellman's reagent, β -hydroxybutyrate dehydrogenase, lipoamide dehydrogenase, D, L-lipoamide and NADH, all incorporated in a carrier matrix. These methods, in general, produce a color the intensity of which is proportionally related to the amount of β-hydroxybutyrate present. The system has the decided drawback that it is not cost effective for general use on an everyday basis, such as is desirable for monitoring weight loss programs.

Another type of known system is the Abbott Laboratories "Medisense" system which measures electrical current changes to determine β-hydroxybutyrate and has been useful in monitoring ketoacidosis conditions of diabetic patients. An attempt to use the device in monitoring a weight loss program, using whole blood samples, is described by Byrne *et al*, *Diabetes Care*, *23*, 500-503 (2000). This system is infeasible for home use because of its instrumentation and whole blood sample requirements. Also, because it produces no color, its results are not as easy to follow as those that involve a color change.

Meanwhile, there are currently many tests available commercially that allow individuals to test themselves at home for numerous other conditions. In general, such tests are in the form of disposable strips comprised of bibulous material that have been pretreated by impregnation with the dry chemistry ingredients requisite to performance of a particular test, requiring no laboratory experience and no instrumentation, which yield a color change upon the application of a few drops of bodily fluid such as urine, saliva or sweat that is noninvasively and readily available to the person conducting the test.

In the field of weight loss management, there are no similar strips available for measuring β-hydroxybutyrate in a test sample such as urine or saliva, both of which can readily be collected in almost any reasonably private milieu by almost any individual. Sweat may also be a useful test sample.

The KetoSite® strips mentioned above measures β-hydroxybutyrate in blood, but they have been found unsuitable for measuring β-hydroxybutyrate in urine for two reasons, i.e.

a) the strips are prepared for a reaction conducted at a pH above 8.5; as a result any sulfhydryl drugs or similar substances in urine will produce false positive results by reacting with tetrazolium salt present in the strip, and

b) the enzyme β-hydroxybutyrate dehydrogenase present in these strips is inhibited by chloride ions, which abound in urine, with the result that false negative results may also be obtained.

Thus it is also apparent that a need exists for a disposable strip that can measure β-hydroxybutyrate in bodily fluid samples that can be obtained noninvasively, such as urine, saliva, or sweat, especially for use in monitoring fat loss as measure of weight.

Brief Description of the Invention

It was unexpectedly found, in the work leading up to the present invention, that if the following reactions, known in the art, are conducted at pH levels that are closer in value to one another and below the pH of at least 8.5(and preferably greater) that is prescribed in the art, a cyclic reaction occurs whereby acetoacetate and β-hydroxybutyrate can both be measured without the two step conversion described, e.g. in the Kojima et al patent referred to above. This is illustrated by a consideration of the following reactions:

Reaction 1:

a) $\beta\text{-hydroxybutyrate} + \text{NAD} \xrightarrow{\beta\text{-hydroxybutyrate dehydrogenase}} \text{Acetoacetate} + \text{NADH}$

b) $\text{NADH} + \text{tetrazolium dye precursor} \xrightarrow{\text{diaphorase}} \text{Reduced tetrazolium dye}$

Reaction 2:

$\text{Acetoacetate} + \text{NADH} \xrightarrow{\beta\text{-hydroxybutyrate dehydrogenase}} \beta\text{-hydroxybutyrate} + \text{NAD}$

By lowering the pH of reaction 1(a) to a pH in the order of from about 7.0 to up to about 8.3 instead of the pH above 8.5 that is prescribed in the art, only a part of the NADH needed in Reaction 1(b) is converted to a purple colored reduced tetrazolium dye because the rate of the reaction between NADH and tetrazolium dye slows in this pH range. A part of the NADH not used up in that reaction accordingly reacts with acetoacetate in the sample, according to Reaction 2, to form to β-hydroxybutyrate + NAD, thus pushing Reaction 1(a) to

proceed further. The overall reaction, initiated by Reactions 1(a) and 1(b), becomes a cyclic one between reactions 1 and 2. The cyclic reaction continues to produce color over a time period and has a substantially increased sensitivity. The preferred tetrazolium dye precursor in this reaction is nitrobluetetrazolium (NBT) which produces a purple color. In the presence of an excess of diaphorase, which acts as an electron mediator to facilitate the formation of reduced tetrazolium dye, the intensity of color formation is proportional to the concentration of β-hydroxybutyrate in the sample. The color formation can easily be quantified by developing a color intensity "key", that can be visually compared to the color produced in a given test. In lieu of a colorimetric measurement, e.g., an electrochemical, spectrophotometric, or fluorimetric measurement, all of which are described in the prior art as appropriate for measuring either NADH or reduced tetrazolium dye, can be utilized. The cyclic reaction can be used to measure acetoacetate and β-hydroxybutyrate, which together account for about 97 to 98% of total ketone bodies. Strips having a reagent layer adapted for this reaction should be used by persons taking sulfhydryl drugs because there is greatly reduced danger of false positive reactions when operating in this pH range. It is noted that this cyclic reaction cannot be used when it is desired to measure total ketone bodies (including acetone) because the nitroprusside and diazonium salts necessary to tie up acetone require higher pH levels in the order of about 8.6 and higher.

Because chloride ion is less prevalent in blood than in urine, it has been found that higher concentrations of β-hydroxybutyrate dehydrogenase, in the order of 10 to 20 times the amount needed in strips designed to be used with blood samples, must be deposited on strips that are intended to be used in measuring β-hydroxybutyrate, whether alone, combined with

acetoacetate, or combined with acetoacetate and acetone, *unless* one uses β-hydroxybutyrate dehydrogenase from a source that is not inhibited by chloride ions.

Chloride ion inhibition can be determined by a simple laboratory test wherein a few drops from a sample solution containing 1 mole each of β-hydroxybutyrate, NAD, β-hydroxybutyrate dehydrogenase and dye is mixed with an equivalent amount per liter of a buffer also containing 1 mole per liter of NaCl, at the pH level of the desired reaction. If color develops, the chloride ion does not inhibit the enzyme β-hydroxybutyrate dehydrogenase; if no color appears, the enzyme is inhibited by the chloride ion.

In tests conducted by the present applicant on β-hydroxybutyrate dehydrogenase from several sources, it was found that this enzyme is not inhibited by chloride ion when obtained from *Alcaligenes,* but no effort was made to make an exhaustive investigation of all possible sources.

When it is desired to measure acetone along with acetoacetate and β-hydroxybutyrate, the addition of nitroprusside or a diazonium salt as a dye is required because these dye precursors react quickly with and tie up acetone in the sample. The effectiveness of attempting to measure acetone, however, must be recognized as virtually nil in all situations where samples are not measured as soon as they are collected, due to the well-known high volatility of this substance.

Still another useful strip, which measures hydroxybutyrate plus acetoacetate in a sample is one impregnated with ingredients for the Reactions (A) and (B) below:

(A) $\beta\text{-hydroxybutyrate} + \text{NAD} \xrightarrow{\beta\text{-hydroxybutyrate dehydrogenase}} \text{Acetoacetate} + \text{NADH}$

In Reaction A, equimolar amounts of acetoacetate and NADH are produced at pH of from about 8.6 to about 9.5. The acetoacetate produced in the reaction and the endogenous acetoacetate in the sample are measured by reaction (B) below, which occurs at pH about 8.6 to about 9.5:

(B) acetoacetate + sodium nitroprusside → magenta color

This strip can be rendered more sensitive by including in it ingredients for also measuring the NADH produced in reaction (A). i.e., a tetrazolium dye precursor and an electron mediator, such as diaphorase. The reaction (C) is as follows:

(C) NADH +tetrazolium salt ⟶ NAD + colored formazan (magenta)

Detailed Description of the Invention

While the various strips referred to herein are especially designed for use with urine samples and other noninvasive samples such as saliva or sweat, in weight loss programs, they may also advantageously be used by persons afflicted with a diabetic, cardiovascular or epileptic condition wherein daily monitoring of ketosis or ketoacidosis is of importance. Also, while they can just as readily be monitored electrochemically, e.g., using one of the convenient

small monitors made for individual use in the home or elsewhere, such as that described in *Byrne et al,* Evaluation of an Electrochemical Sensor for Measuring Blood Ketones, 23 *Diabetes Care* 500-503 (April 2000), they may also be measured in a fluorimeter or spectrophotometer as described in various of the prior publications referred to herein above.

The strips prepared in accordance with this invention can employ any convenient porous paper or membrane as its reagent layer. This porous material, after impregnation with the requisite chemicals, is applied to a less porous, more durable backing material, referred to as the support layer. This layer protects the integrity of the dried impregnated porous strip during packing, transportation and storage from forces of wear and tear and permits the strip to be more easily handled by the user after it is wetted with the sample. In addition, the support layer also protects the integrity of the reagent layer during wetting with sample and reading of the result.

In general the strips are prepared by impregnating the reagent layer with all of the necessary ingredients and a buffer that has a pH level commensurate with the pH at which the reaction between reagents and sample is desired to occur. The reagent layer is then dried in a suitable drying apparatus. Alternatively, the reagent layer could be sprayed on the reagent layer before or after it is affixed to the backing. The assembly may be cut into strips before impregnation or after drying, depending on the equipment available. Any other order or mode of assembly of the layers that includes a step of either impregnation of the reagent layer or deposition on the layer and a drying step and is effective to ensure that strips each containing a dried reagent layer affixed to a backing layer are produced may be utilized.

Ideally, the strips will be availed of on a daily basis by people on weight loss diets or persons with disease states in which such monitoring is advisable. The strips of this invention afford great advantages to people on weight loss diets because all embodiments described herein measure β-hydroxybutyrate which typically comprises 75-80% of total ketone bodies. The embodiments which measure both acetoacetate and β-hydroxybutyrate measure in the order of 97-98% of total ketone bodies, and of course, the embodiment which measures total ketone bodies gives what is essentially a 100% result, provided the measurement is made promptly after collection of the sample so that acetone content does not volatilize before measurement can occur. The ability to make these measurements of β-hydroxybutyrate, with or without acetoacetate and acetone, in urine, saliva or sweat samples is likewise highly advantageous, especially when daily monitoring is desirable or indicated. Collecting blood samples, as has been required for β-hydroxybutyrate measurement heretofore, is not only invasive but if done at frequent intervals is extremely uncomfortable for many people and certainly should not be a requisite for people seeking to monitor a weight loss diet. To patients whose diabetic, cardiovascular or epileptic conditions require daily or very frequent monitoring, the strips of the present invention with their ability to measure β-hydroxybutyrate alone, or combined with acetoacetate, or further combined with acetone in urine rather than blood should offer a great boon.

The present invention, in essence, provides the possibility of assaying for β-hudroxybutyrate alone in a sample, using a test strip that provides β-hydroxybutyrate dehydrogenase, NAD and a tetrazolium dye precursor, at a pH of about 8.6 or higher, up to about 9.5, plus a minor amount of an electron mediator capable of transferring an electron to

the dye precursor to effect a color change. In this embodiment, the use of β-hydroxybutyrate dehydrogenase (1) from an *Alcaligenes* source, or another source such that this enzyme is not inhibited by chloride ions in the sample, or alternatively, (2) the addition to the strip of an excess, in the order of at least 10 and up to about 20 times the amount required in strips designed primarily for use with blood samples, of β-hydroxybutyrate dehydrogenase, obtained from a source that *is* inhibited by chloride ions in the sample, such as *Pseudomonas,* assures that the strip will measure β-hydroxybutyrate in urine samples.

When the strip is intended to measure both β-hydroxybutyrate and acetoacetate in the sample, the reagent layer of the strip maybe impregnated in three alternative ways:

(1) It may again contain β-hydroxybutyrate dehydrogenase, NAD, a tetrazolium dye precursor and an electron mediator, but it must be impregnated also with buffer at a pH level that ensures that the reaction with the sample will occur at a pH from 7.0 up to about 8.3, so that the cyclic reaction proceeds, whereby (i) β-hydroxybutyrate + NAD is converted to acetoacetate + NADH, (ii) the reaction of NADH with tetrazolium dye precursor to produce reduced tetrazolium dye is slowed and (iii) unused NADH then commences converting endogenous acetoacetate in the sample to β-hydroxybutyrate + NAD. In this embodiment, one of the alternative precautions relative to β-hydroxybutyrate dehydrogenase discussed above must also be observed in preparing the reagent layer of the strip.

(2) The second embodiment of this strip may have in its reagent layer NAD, β-hydroxybutyrate dehydrogenase and sodium nitroprusside. The buffer employed in impregnating the strip must be adjusted to a pH level such that the reaction will proceed at a pH of from about 8.6 to about 9.5. Here, too, one of the alternative precautions relative to the origin of the β-hydroxybutyrate dehydrogenase that is necessary to enable urine samples to be tested must be observed.

(3) The third embodiment of this strip may have in its reagent layer NAD, β-hydroxybutyrate dehydrogenase, sodium nitroprusside, a tetrazolium dye precursor and an electron mediator. The buffer employed in impregnating the strip must be adjusted to a pH level such that the reaction will proceed at a pH of from about 8.6 to about 9.5. Here again one of the alternative precautions relative to β-hydroxybutyrate origin must be observed.

The test strip for measuring total ketone bodies (TKB), including acetone, must contain either nitroprusside or a diazonium salt. The second and third alternatives above are both suitable *per se* for this purpose, providing a sufficient amount of nitroprusside is included, so that it ties up acetone virtually instantaneously upon contacting the sample, while leaving sufficient unreacted sodium nitroprusside to react with the endogenous acetoacetate and that converted from β-hydroxybutyrate in the sample.

In the foregoing discussion of the possible reagent layers for various assay purposes, it is to be understood that where a tetrazolium dye precursor is specified, various such precursors may be utilized. They include at least 2(2'benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl)

tetrazolium (BSPT), 2-benzothiazolyl-(2)-3, 5-diphenyl tetrazolium (BTDP), 2,3-di(4-nitrophenyl) tetrazolium (DNP), 2,5-diphenyl-3-(4-styrylphenyl) tetrazolium (DPSP), distyryl nitroblue tetrazolium (DS-NBT), 2-(indophenyl)-3-(paranitrophenyl)-5-phenyl-tetrazolium chloride(INT), 3,3'-[3,3'-dimethoxy-(1,1'-biphenyl)-4,4'-diyl]-bis[2-(4-nitrophenyl)-5-phenyl(-2H tetrazolium (NBT), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium (MTT), 2-phenyl-3-(4-carboxyphenyl)-5-methyl tetrazolium (PCPM), tetrazolium blue (TB), thiocarbamyl nitroblue tetrazolium (TCNBT), tetranitroblue tetrazolium (TNBT), tetrazolium violet, (TV), 2-benzothiazothiazoyl-3-(4-carboxy-2-methoxyphenyl)-5-[4-(2-sulfoethylcarbamoyl)phenyl]-2H-tetrazolium (WST-4), and 2,2'-dibenzothiazolyl-5,5'bis[4-di(2-sulfoethyl)carbamoylphenyl]-3,3'-(3, 3'-dimethoxy-4,4'-biphenylene)ditetrazolium, and disodium salt (WST-5).

The diaphorase enzyme referred to as an electron mediator may be a lipoic dehydrogenase, a ferredoxin-NADP-reductase or a lipoamide dehydrogenase. Alternatively to adding a diaphorase enzyme, phenazinium methyl sulfate or 1-methoxyphenazinium methyl sulfate may also be used.

A particularly suitable diazonium salt for use in a TKB assay reagent layer is 4-nitrobenzene diazonium tetrafluoroborate.

In lieu of employing a strip, the cyclic reaction described herein may be conducted with sample and a reagent mix of NAD, β-hydroxybutyrate dehydrogenase, tetrazolium dye precursor and electron mediator, such as diaphorase, in a buffer having a pH of from about 7.0 up to about 8.3 in a typical laboratory setup and the color produced may be measured in a fluorimeter or spectrophotometer. Other possible ways of employing this cyclic reaction

beneficially are in an automatic analyzer such as used in the Kojima patent discussed above where a spectrophotometric measurement is made, or in a system similar to the Abbott "Medisense" discussed above where an electrochemical measurement is made. Of course, a visual color standard can always be established and used if desired.

In using strips as herein described, each strip should be dipped in the sample momentarily and then set aside and allowed to rest briefly (about ½ minute to about 5 minutes) to ensure that the reaction proceeds to completion before the strip is read.

The following examples illustrate the preparation of strips for particular assays and compare the performance of the strips to one another and to strips previously available in the art. In these examples, wherever "U" appears, it is to be understood that "U" defines a unit of enzyme activity necessary to reduce 1 micromol of NAD to NADH per minute at a temperature of 30°C.

EXAMPLE #1

A Method and Strip Device to Measure Total Ketone Bodies, i.e. β-hydroxybutyrate, Acetoacetate an Acetone As One Step

The formulation contains β-hydroxybutyrate dehydrogenase enzyme (HBD) and NAD which converts β-hydroxybutyrate to acetoacetate at pH 8.5. The "converted acetoacetate" thus produced and endogenous acetoacetate in the sample, plus acetone, are measured by a nitroprusside reaction. The reactions are:

$$\beta\text{-hydroxybutyrate} + \text{NAD} \xrightarrow{\beta\text{-hydroxybutyrate dehydrogenase}} \text{Acetoacetate} + \text{NADH}$$

$$\text{Acetoacetate (Converted + Endogenous)} + \text{acetone} \xrightarrow{\text{Nitroprusside at pH 8.6}} \text{Magenta color}$$

The following ingredients were mixed:

Tris-HCl (Buffer), pH 8.6	1M
β-hydroxybutyrate dehydrogenase	100U/mL (about 2.0 to 2.5 U per strip)
NAD	3%
Sodium Nitroprusside	5%
Magnesium sulfate heptahydrate	30%

To make the strip, filter paper, in this case, Whatman-54 (though other porous papers may be substituted) was immersed in the above formulation, removed, and then dried in an oven at 45°C for 20 minutes. The strips were made by sticking a ¼" layer of said dried paper on the bottom of a polystyrene card having the dimensions 3" by 12", with the help of double adhesive tape. The card was then cut lengthwise into 48 strips, each ¼" x 3". These strips, which measure Total Ketone Bodies in human bodily fluids are referred to as "TKB" and their use in a weight loss program is demonstrated in Example 6.

Instead of nitroprusside, a known diazonium salt, such as 4-nitrobenzene diazonium tetrafluoroborate, can be substituted.

In other experimental work, strips containing concentrations of β-hydroxybutyrate dehydrogenase up to 300 U/mL have been employed.

EXAMPLE 1B

A Method and Device to Measure Total Ketone Bodies As One Step with Increased Sensitivity

The formulation contains:

Tris Buffer, pH 8.6	0.1M
β-hydroxybutyrate dehydrogenase	300U/mL
NAD	3%
Sodium Nitroprusside	5%
Magnesium sulfate heptahydrate	30%
Diaphorase	100 U/mL
NBT	2mM

The filter paper such as Whatman-54 is dipped in the above formulation and is dried in the oven at 45°C for 20 minutes. The strips are made by sticking a 1/4" of layer of said paper on the bottom of the polystyrene card which is 12" long and 3" high with the help of double adhesive tape. The card is cut lengthwise into 48 strips of 1/4" x 3" high strips. These strips are more sensitive in measurement of Total Ketone Bodies (TKB) than those shown in Example 1.

Similarly to example 1, this formulation contains β-hydroxybutyrate dehydrogenase enzyme (HBD) and NAD which at pH 8.6 converts β-hydroxybutyrate to acetoacetate and NADH on an equimolar basis (Reaction 4) and then "converted acetoacetate" from reaction 1 and endogenous acetoacetate and acetone in the sample are measured by nitroprusside as shown in reaction B above.

By adding NBT and diaphorase to this same formulation, NADH obtained from reaction A discussed above, is also converted to color on an equimolar basis as shown in reaction C above, thereby further increasing the sensitivity of the test.

EXAMPLE #2

A Method and Device To Measure β-hydroxybutyrate and Acetoacetate Simultaneously In a "cyclic" Fashion.

The formulation contains β-hydroxybutyrate dehydrogenase, NAD, NBT and diaphorase at pH 8.0.

To make the strips, the following ingredients were first mixed:

Tris-HCl, pH 8.0	0.1M
β-hydroxybutyrate dehydrogenase	100 U/mL (about 4 U/strip)
NAD	3%
NBT	0.2%
Diaphorase	10 U/mL
Magnesium chloride	0.1%
Surfonyl (a surfactant)	0.06%

Whatman-54 filter paper was immersed in the above formulation, removed and dried in the oven at 45°C for 20 minutes. The strips were made by sticking a ¼" layer of said dried paper on the bottom of a polystyrene card of 12" by 3" dimension with the help of double adhesive tape. The card was cut lengthwise into 48 strips of ¼" x 3" strips. These strips were used for testing human bodily fluids. These strips, which measure β-hydroxybutyrate plus acetoacetate are referred to as "HB&AA", and their use in weight loss program is demonstrated in example 6.

EXAMPLE #3

A Method and Device To Measure β-hydroxybutyrate Alone In Serum (blood) Samples Obtained From Weight Loss Program That Uses Normal Concentration Of β-HBD, Similar To The Device Available Commercially As KetoSite® From GDS Technology, Inc.

The formulation contains a **normal level of** β-hydroxybutyrate dehydrogenase according to the prior art (0.2-5.0 U/mL), NAD, NBT and diaphorase at pH 8.6.

β-hydroxybutyrate dehydrogenase (Pseudomonas)	15 U/mL (about 0.2 U per strip)
NAD	3%
NBT	0.2%
Diaphorase	30 U/mL
Magnesium chloride	0.1%
Surfonyl	0.05%
Tris-HCl, (Buffer) pH 8.6	0.1 M

Whatman-54 filter paper was immersed in the above formulation, removed and dried in the oven at 45°C for 20 minutes. The strips were made by sticking a ¼" layer of said dried paper on the bottom of a polystyrene card of 12" long by 3" dimension, with the help of double adhesive tape. The card was cut lengthwise into 48 strips of ¼" x 3" strips. These strips were used for testing of human biological fluids. These strips, which measure hydroxybutyrate alone using what was heretofore characterized as a "normal" concentration of β-hydroxybutyrate dehydrogenase are referred to as "HB-L".

As demonstrated in Table 1, it was surprisingly found that both HB-L strips and commercial KetoSite strips can be used to measure β-hydroxybutyrate by dipping each of them in serum (blood) and then allowing the saturated strip to rest for one minute. The samples were obtained from people on a weight-loss program. The relative intensity of purple color is indicated by the number "+" signs and absence of color is indicated by "-" signs.

Table 1

Serum Samples with β-hydroxybutyrate	**HB-L Strips**	**KetoSite**
1. 0.12 mM conc	+	+
2. 0.25 mM conc	++	++
3. 0.52 mM conc	+++	+++
4. 1.14 mM conc	++++	++++
5. 2.5 mM conc	++++++	++++++

In contrast to serum samples as shown in Table 1, Table 2 shows that both strips, when dipped in urine containing similar levels of concentration of β-hydroxybutyrate either did not show any color, after resting for one minute or showed a very light color at the higher concentrations of β-hydroxybutyrate. The relative intensity of purple color is again indicated by "+" signs and absence of color is indicated by "-" signs.

Table 2

Urine samples with β-hydroxybutyrate concentration	HB-L strip	KetoSite
1. 0.11 mM	- (Negative)	- (Negative)
2. 0.22 mM	-	-
3. 0.48 mM	-	-
4. 1.12 mM	-	-
5. 2.22 mM	++	+

EXAMPLE #4

A Method And Device Of Measuring β-hydroxybutyrate Alone In Urine With A Strip Having A High Level Of β-hydroxybutyrate Dehydrogenase And Other Components In A Formulation Similar To That Shown In Example 3.

The following ingredients were mixed:

β-hydroxybutyrate dehydrogenase (origin: Pseudomonas)	200 U/mL (about 4U/strip)
NAD	3%
NBT	0.2%
Diaphorase	30 U/mL
Magnesium chloride	0.1%
Surfonyl	0.05%
Tris-HCl, (Buffer) pH 8.6	0.1 M

Whatman-54 filter paper was immersed in the above formulation, removed and dried in the oven at 45°C for 20 minutes. The strips were made by sticking a ¼" layer of said paper on the bottom of a polystyrene card of 12" by 3" dimension, with the help of double adhesive tape. The card was cut lengthwise into 48 strips of ¼" x 3" high strips and the strips were used for testing human bodily fluids. These strips with a high concentration of β-hydroxybutyrate dehydrogenase in order of 4 U per strip are referred to as "HB-H". As shown in Table 3, using "HB-H" strip and urine samples of the same origin as those in Table 2, substantially improved sensitivity to β-hydroxybutyrate was obtained. Use of this HB-H strip in a weight loss program is demonstrated in Example 6.

Table 3

Urine samples with β-hydroxybutyrate	**HB-H Strip**	**HB-L strip**	**KetoSite (commercial)**
1. 0.11 mM	+	- (Negative)	- (Negative)
2. 0.22 mM	++	-	-
3. 0.48 mM	+++	-	-
4. 1.12 mM	++++	-	-
5. 2.22 mM	+++++	+	+

EXAMPLE #5

A Method Of Measuring β-hydroxybutyrate In Urine Using β-hydroxybutyrate Dehydrogenase Enzyme (origin: Alcaligenes) Which Is Not Inhibited By Chloride Ions.

The following were mixed:

β-hydroxybutyrate dehydrogenase (from Alcaligenes)	15U/mL (about 0.3 U per strip)
NAD	3%
NBT	0.2%
Diaphorase	30 U/mL
Magnesium chloride	0.1%
Surfonyl	0.05%
Tris-HCl, pH 8.6	0.1 M

Whatman-54 filter paper was immersed in the above formulation, removed and dried in the oven at 45°C for 20 minutes. The strips were made by sticking a dried ¼" layer of said paper on the bottom of a polystyrene card of 12" long by 3" high with the help of double adhesive tape. The card was cut lengthwise into 48 strips of ¼" x 3" high strips and the strips were used for testing human bodily fluids. These strips using β-hydroxybutyrate dehydrogenase that is insensitive to chloride ions were referred to as HB-L-A". HB-L-A strips containing "normal" concentration of this particular β -hydroxybutyrate dehydrogenase were able to detect β-hydroxybutyrate in urine, similarly to the HB-H strips and in contrast to the HB-L strip or the KetoSite strip (Table 4). The color was measured one minute after dipping in and removal from urine.

Table 4

Urine samples with β-hydroxybutyrate	HB-L-A strip	HB-H Strip	HB-L strip	KetoSite
1. 0.11 mM	+	+	- (Negative)	- (Negative)
2. 0.22 mM	++	++	-	-
3. 0.48 mM	+++	+++	-	-
4. 1.12 mM	++++	++++	-	-
5. 2.22 mM	+++++	+++++	+	+

Tables 3 and 4 show that the alternative corrections to the formula for the inhibitive effect of chloride ion (i.e. use of β-HBD from a source that is uninhibited by chloride, as in H-B-L-A, or increasing the concentration of β-HBD from a source that *is* inhibited by chloride ion as in HB-H) are effective in making these strips very sensitive.

EXAMPLE #6

Utility of Strips in Weight Loss Program

The strips TKB (described in Example 1) for measuring Total Ketone bodies, HB&AA (described in Example 2) for measuring both β-hydroxybutyrate and acetoacetate in one step in a cyclic method, HB-H (as described in Example 4) for measuring β-hydroxybutyrate alone, and commercially available strips for measurement of acetoacetate (AA), KetoSite® from Bayer Diagnostics, Elkhart, Indiana were used in a weight loss program. These strips were used for twenty days, each morning, on samples from persons who were on various 1000-1500 calorie diets. These strips were quickly dipped in urine and removed, and color was visually measured in a semi-quantitative fashion after one minute. Diet 1 contained approximately 30%

carbohydrates, 40% fat, and 30% protein and results are shown in Table 5. Diet 2 contained approximately 40% carbohydrate, 30% fat and 30% protein and results are shown in Table 6. Diet 3 contained approximately 50% carbohydrates, 20-25% fat, and 20-25% protein and results are shown in Table 7. Diet 4, similar to the Atkins diet, was low in carbohydrate and high in fat containing approximately 10% carbohydrates, 40-50% fat, 30-40% protein and the results are shown in Table 8. As demonstrated in Tables 5, 6, 7, and 8, all three strips of this invention (TKB, HB&AA, HB-H) showed a positive color with a low level of ketone bodies whereas the commercially available strips, which measure only acetoacetate and acetone (AA), were negative except on samples taken from persons on Diet 4. All three strips showed a higher intensity of color, as compared to the AA strip, with samples of urine from persons on a high fat, low carbohydrate diet (Table 8).

TABLE #5 *with* Diet #1

Sample	TKB Strip	HH &AA Strip	HB-H Strip	AA Strip
1	+	+	+	-
2	+	+ +	+	-
3	+ +	+ + +	+	-
4	+ +	+ + +	+ +	+
5	+ +	+ +	+	-
6	+ +	+ +	+	-
7	+ +	+	+	-
8	+ +	+ +	+	-
9	+	+ +	+	-
10	+ +	+ + +	-	+
11	+	+	+	-
12	+ +	+ +	+	-
13	+	+	+	-
14	+	+ +	+	-
15	+ +	+	+	-
16	+ +	+	+	-
17	+ +	+ +	+	-
18	+	+	-	-
19	+ +	+ +	+	-
20	+	+	-	-

TABLE #6 *with* Diet #2

Sample	TKB Strip	HH &AA Strip	HB-H Strip	AA Strip
1	+	+	+	-
2	+	++	++	-
3	++	+++	++	-
4	++	+++	++	-
5	++	++	++	-
6	++	++	++	-
7	++	+	+	-
8	++	++	+	-
9	+	++	+	+
10	++	+++	+	-
11	+	+	-	-
12	++	++	+	-
13	+	+	+	-
14	+	++	+	-
15	+	+	+	-
16	+	+	+	-
17	+	++	+	-
18	+	+	-	-
19	+	++	+	-
20	+	+	+	-

TABLE #7 *with* Diet #3

Sample	TKB Strip	HH &AA Strip	HB-H Strip	AA. Strip
1	+	+	-	-
2	+	+	-	-
3	++	++	+	-
4	+	++	+	-
5	++	++	+	-
6	+	++	+	-
7	++	+	+	-
8	++	++	+	-
9	+	++	+	+
10	+	+	+	-
11	+	+	+	-
12	++	++	+	-
13	+	++	+	-
14	+	++	+	-
15	++	+	+	-
16	++	+	+	-
17	++	++	+	-
18	+	+	-	-
19	++	++	+	-
20	+	+	-	-

TABLE #8 *with* Diet #4

Sample	TKB Strip	HH &AA Strip	HB-H Strip	AA Strip
1	+	+	+	-
2	+ + +	+ + +	+ +	+
3	+ + +	+ + +	+ +	+
4	+ + + +	+ + + +	+ + +	+ +
5	+ + + +	+ + + +	+ +	+ +
6	+ +	+ +	+ +	+
7	+ + +	+ + +	+	+ +
8	+ + +	+ + +	+ +	+
9	+ +	+ +	+	+
10	+ +	+ +	+	+
11	+ +	+ +	+	-
12	+ +	+ +	-	+
13	+	+ +	+	+
14	+	+ +	+	+
15	+ +	+ +	+	-
16	+ +	+ +	+	+
17	+ +	+ +	+	-
18	+ +	+ +	+	-
19	+ +	+ +	+	-
20	+ +	+ +	+	-

Obviously, many modifications and variations of the invention as hereinbefore set forth can be made without departing from the spirit and scope thereof. It is accordingly intended that it be limited only by the claims hereof.